Telomerase expression by aberrant methylation of the TERT promoter in melanoma arising in giant congenital nevi

Fan, Yiping ; Lee, Seungjae ; Wu, Gang ; Easton, John ; Yergeau, Donald ; Dummer, Reinhard ; Vogel, Peter ; Kirkwood, John M ; Barnhill, Raymond L ; Pappo, Alberto ; Bahrami, Armita

DOI: https://doi.org/10.1038/jid.2015.374

Telomerase Expression by Aberrant Methylation of the *TERT* Promoter in Melanoma Arising in Giant Congenital Nevi

Journal of Investigative Dermatology (2016) **136,** 339–342; doi:10.1038/JID.2015.371

TO THE EDITOR

Telomeres are tandem repeats of the noncoding DNA structures at the end of human chromosomes that protect the coding DNA and the integrity of the genome (Blackburn, 1991). The ability to sustain telomere length confers unlimited proliferative capacity to cancer cells. In most cancers telomere length is maintained by the activity of the enzyme telomerase (Kim et al., 1994), whose catalytic subunit is encoded by the telomerase reverse transcriptase (*TERT*) gene. However, until recently, the underlying mechanisms for telomerase activation in cancer cells were largely unknown.

Recurrent transcription activating mutations of the *TERT* promoter were first described in melanoma and subsequently in other tumor types (Horn et al., 2013; Vinagre et al., 2013). These mutations upregulate *TERT* expression by recruiting the multimeric GA-binding protein transcription factor that specifically binds to the mutant promoter (Bell et al., 2015). In addition to the mutations, DNA methylation of the *TERT* promoter is likely to play a role in *TERT* expression (Guilleret and Benhattar, 2004). More recently it was shown that a region of the *TERT* promoter upstream of the transcription start site is methylated in malignant telomerase-expressing pediatric brain tumors but not in telomerase-negative normal brain tissue or low-grade tumors (Castelo-Branco et al., 2013).

We previously showed that an aggressive form of pediatric melanoma developing within giant congenital nevi (GCN) retains the wild-type *TERT* promoter (Lu et al., 2015). To determine whether epigenetic modifications may play a role in telomerase expression in this melanoma subtype, we analyzed the DNA methylation profile of a CpG-rich region of the TERT promoter, shown previously to be differentially methylated between normal and malignant tissues (Castelo-Branco et al., 2013), in 13 melanomas (3 arising in GCN, 7 conventional, and 3 spitzoid) and 10 benign or borderline melanocytic tumors (1 GCN, 3 GCN with nodular proliferation, and 6 borderline spitzoid melanocytic neoplasms) from 23 pediatric and adult patients. The human investigations were performed after approval by local institutional review boards. Written, informed patient consent was waived because the research involved no more than minimal risk to the subjects. The status of the *TERT* promoter, *BRAF* and *NRAS* mutations, and kinase fusions was available for the spitzoid tumors and a subset of melanoma samples from our prior studies (Lee et al., 2015; Lu et al., 2015).

Supplementary Table S1 (online) and Figure 1 summarize the demographic and outcome data and the primary driver oncogene for the 23 study subjects. PCR–Sanger sequencing identified a hotspot *TERT* promoter mutation (4 C228T [chr5:1,295,228], 3 C250T [chr5: 1,295,250], and 2 CC242/243TT [chr5: 1,295,242-3]) in 9 of 13 melanoma samples (6/7 conventional; 3/3 spitzoid; 0/3 melanomas in GCN) but not in the 10 benign or borderline melanocytic neoplasms (Figure 1). The DNA methylation status of a region of the *TERT* promoter, from 482 bp to 667 bp upstream of the ATG start site (chr5: 1295586–1295771 [GRCh37/hg19]) (see Supplementary Figure S1, online), encompassing 26 CpG sites, was assayed by next-generation bisulfite sequencing (see Supplementary Methods, online). For each CpG site, the methylation ratio (beta value) was measured in the range of 0 to 1 (see Supplementary Table S2, online). The methylation status was defined as follows: >0.7, methylated (Figure 1, red); 0.5 to 0.7, partially methylated (orange); 0.3 to <0.5, partially unmethylated (cyan); and <0.3, unmethylated (blue). Supplementary Table S3 (online) shows the total number of methylated Cs and unmethylated Cs in the sequenced region for each sample. Remarkably, almost all 26 CpG sites in the sequenced region were highly methylated in the three melanomas arising in GCN (S1, S2, S21) and in the one conventional melanoma bearing wild-type *TERT* promoter (S22), whereas the CpG sites remained predominantly unmethylated in the 9 mutant *TERT* promoter melanomas and the 10 benign or borderline melanocytic neoplasms (Figure 1).

Next, we evaluated the association of *TERT* promoter CpG methylation with telomerase expression by *TERT* mRNA in situ hybridization and by gene expression analysis (Supplementary Methods). *TERT* mRNA in situ hybridization revealed distinct, intracellular punctate signals in melanomas arising in GCN (Figure 2c and f) but not in the proliferative nodules in GCN (Figure 2i and l). The *TERT* promoter methylation level was calculated as the log 2 ratio of the total number of methylated Cs versus the total number of unmethylated Cs in the sequenced region (logit [beta value]). The *TERT* expression level was measured by using RNA sequencing data available for a subset of samples. An association analysis revealed a strong correlation between *TERT* promoter methylation and *TERT* expression level ($P = 0.0422$, adjusted $r^2 = 0.5145$; see Supplementary Figure S2, online).

Our data demonstrate that epigenetic modification through *TERT* promoter CpG methylation is an alternative pathway for *TERT* reactivation in melanoma. Although epigenetic remodeling by promoter methylation is

Abbreviations: GCN, giant congenital nevi; TERT, *telomerase reverse transcriptase (gene)*

Accepted manuscript published online 24 September 2015

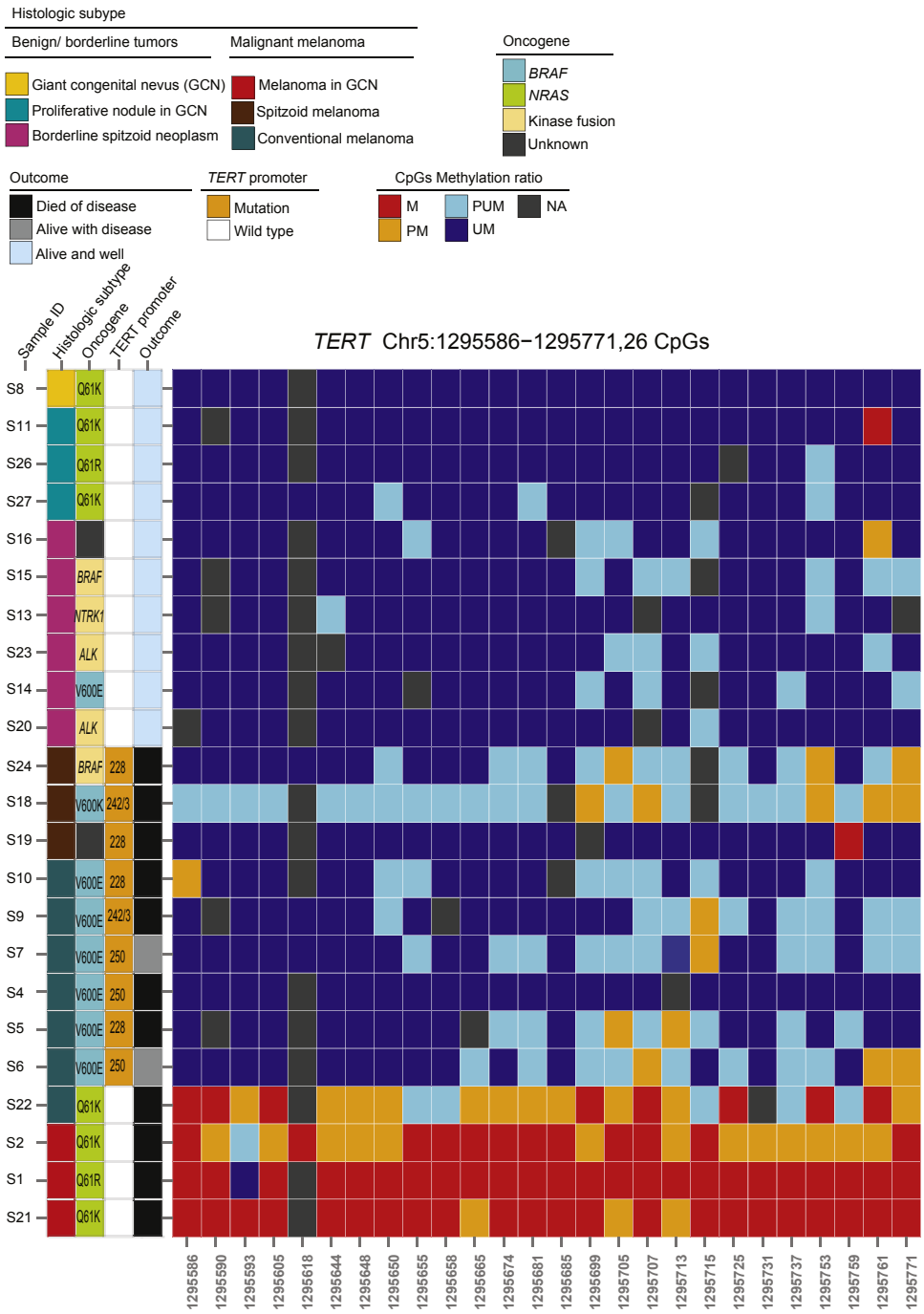

Figure 1. Association of the mutational status and the methylation profile of the *TERT* promoter with disease characteristics and outcome data for 23 patients with melanocytic tumors. The 26 CpG sites were aberrantly methylated in wild-type *TERT* promoter melanomas (the last four rows) but were predominantly unmethylated in low-grade or benign melanocytic tumors (atypical spitzoid neoplasms and GCN with proliferative nodules) and in mutant *TERT* promoter melanomas. Methylation panel color code: M, methylated; PM, partially methylated; PUM, partially unmethylated; UM, unmethylated; NA, not available.

generally considered a signature of gene silencing, *TERT* expression is paradoxically increased by promoter methylation (Guilleret and Benhattar, 2004). Although the exact mechanism underlying CpG DNA methylation in *TERT* upregulation is not known, one possible mechanism is by inhibiting transcriptional repressors such as CTCF (Renaud et al., 2007), SIN3A , or MAZ (Xu et al., 2013) from binding to the target DNA-binding sites in the region (Supplementary Figure S1). Also, even when the promoter is largely methylated, a small region of the core promoter upstream of the transcription start site remains unmethylated to allow for the continued transcriptional activity of *TERT* (Renaud et al., 2007; Zinn et al., 2007).

Individuals with GCN are at increased risk for developing melanoma (Figure 2a, b, d, and e) that occurs most frequently in the first decade of life (Bittencourt et al., 2000). A much more frequent change in these nevi than melanoma is the development of clonal

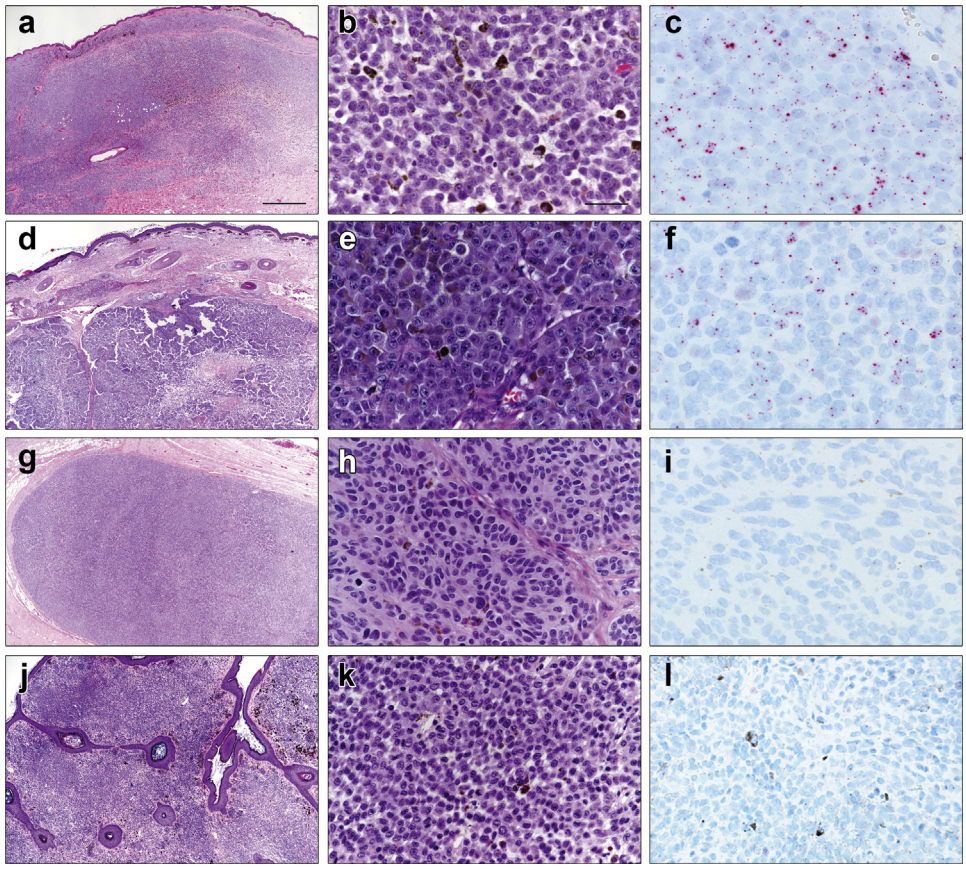

Figure 2. Representative photomicrographs of H&E-stained histological sections and *TERT* mRNA in situ hybridization. The two top panels represent two melanomas in GCN and the two bottom panels represent two proliferative nodules in GCN. mRNA in situ hybridization shows numerous high-resolution red intracellular punctate signals in malignant melanocytes (**c** and **f**) and no signals above the background level in melanocytes of proliferative nodules (**i** and *l*). Scale bars = 1000 μm (**a**) and 50 μm (**b**).

proliferations often in the form of nodules (Figure 2g, h, j, and k), which may suggest or mimic melanoma on clinical or histologic grounds (Yelamos et al., 2015). The differential pattern for *TERT* promoter methylation and telomerase expression between melanomas in GCN and proliferative nodules demonstrated in our study is consistent with the benign clinical course of proliferative nodules compared with the invariably aggressive behavior of melanoma arising in GCN. Further studies in a larger number of patients are needed to determine the potential diagnostic value of *TERT* promoter methylation assays for ambiguous proliferative lesions within GCN.

In our cohort, *TERT* promoter hypermethylation or promoter mutations occurred in all melanoma samples but in none of the benign or borderline melanocytic lesions, suggesting that a panel incorporating *TERT* promoter methylation and mutation assays may help discriminate between benign/borderline and overtly malignant melanocytic neoplasms. Future studies need to assess the potential use of these assays for diagnostic or prognostic purposes in the clinic.

In summary, we demonstrate that in subsets of malignant melanoma, *TERT* is upregulated epigenetically by a methylation-dependent mechanism. These findings have potential therapeutic implications. *TERT* promoter CpG hypermethylation is a reversible phenomenon. Treatment with DNA demethylating agents reduced *TERT* expression and telomerase activity in telomerase-positive cell lines (Guilleret and Benhattar, 2003; Renaud et al., 2007). Together, these findings provide a rationale for developing a therapeutic strategy in preclinical studies through epigenetic modifications at *TERT* promoter regulatory sites in melanomas with the CpG island methylator phenotype.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This research was supported in part by the National Cancer Institute of the National Institutes of Health under Award Number P30CA021765 and by ALSAC.

***Yiping Fan*[1,2], *Seungjae Lee*[2,3], *Gang Wu*[1], *John Easton*[1], *Donald Yergeau*[1], *Reinhard Dummer*[4], *Peter Vogel*[3], *John M. Kirkwood*[5], *Raymond L. Barnhill*[6], *Alberto Pappo*[7] and *Armita Bahrami*[3,*]**

[1]*Department of Computational Biology, St. Jude Children's Research Hospital, Memphis, Tennessee;* [3]*Department of Pathology, St. Jude Children's Research Hospital, Memphis, Tennessee;* [4]*Department of Dermatology, University Hospital Zurich, Zurich, Switzerland;* [5]*Division of Hematology-Oncology, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania;* [6]*Departement of Pathology, Institute Curie, Paris, France and* [7]*Department of Oncology, St. Jude Children's Research Hospital, Memphis, Tennessee*

[2] *These authors contributed equally to this work.*

[*]*Corresponding author e-mail: armita.bahrami@stjude.org*

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1038/JID.2015.374.

REFERENCES

Bell RJ, Rube HT, Kreig A, et al. Cancer. The transcription factor GABP selectively binds and activates the mutant TERT promoter in cancer. Science 2015;348:1036–9.

Bittencourt FV, Marghoob AA, Kopf AW, et al. Large congenital melanocytic nevi and the risk for development of malignant melanoma and neurocutaneous melanocytosis. Pediatrics 2000;106:736–41.

Blackburn EH. Structure and function of telomeres. Nature 1991;350:569–73.

Castelo-Branco P, Choufani S, Mack S, et al. Methylation of the TERT promoter and risk stratification of childhood brain tumours: an integrative genomic and molecular study. Lancet Oncol 2013;14:534–42.

Guilleret I, Benhattar J. Demethylation of the human telomerase catalytic subunit (hTERT) gene promoter reduced hTERT expression and telomerase activity and shortened telomeres. Exp Cell Res 2003;289:326–34.

Guilleret I, Benhattar J. Unusual distribution of DNA methylation within the hTERT CpG island in tissues and cell lines. Biochem Biophys Res Commun 2004;325:1037–43.

Horn S, Figl A, Rachakonda PS, et al. TERT promoter mutations in familial and sporadic melanoma. Science 2013;339:959–61.

Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011–5.

Lee S, Barnhill RL, Dummer R, et al. TERT promoter mutations are predictive of aggressive clinical behavior in patients with spitzoid melanocytic neoplasms. Sci Rep 2015;5:11200.

Lu C, Zhang J, Nagahawatte P, et al. The genomic landscape of childhood and adolescent melanoma. J Invest Dermatol 2015;135:816–23.

Renaud S, Loukinov D, Abdullaev Z, et al. Dual role of DNA methylation inside and outside of CTCF-binding regions in the transcriptional regulation of the telomerase hTERT gene. Nucleic Acids Res 2007;35:1245–56.

Vinagre J, Almeida A, Populo H, et al. Frequency of TERT promoter mutations in human cancers. Nat Commun 2013;4:2185.

Xu M, Katzenellenbogen RA, Grandori C, et al. An unbiased in vivo screen reveals multiple transcription factors that control HPV E6-regulated hTERT in keratinocytes. Virology 2013;446:17–24.

Yelamos O, Arva NC, Obregon R, et al. A comparative study of proliferative nodules and lethal melanomas in congenital nevi from children. Am J Surg Pathol 2015;39:405–15.

Zinn RL, Pruitt K, Eguchi S, et al. hTERT is expressed in cancer cell lines despite promoter DNA methylation by preservation of unmethylated DNA and active chromatin around the transcription start site. Cancer Res 2007;67:194–201.

Diabetic Wounds Exhibit Distinct Microstructural and Metabolic Heterogeneity through Label-Free Multiphoton Microscopy

Journal of Investigative Dermatology (2016) **136,** 342–344; doi:10.1038/JID.2015.371

TO THE EDITOR

Cutaneous wound healing is a complex process requiring the coordination of inflammatory cells, endothelial cells, fibroblasts, and keratinocytes (Gurtner et al., 2008). Chronic wounds fail to progress through this coordinated process, and are often characterized by prolonged inflammation, poor vascularization, callus formation, and infection (Brem et al., 2007; Martin et al., 2010). A wide variety of advanced wound care products have been developed to target specific characteristics of chronic wounds (Apelqvist, 2012). However, no single therapy has yielded widespread clinical success, and there is a critical need to develop new biomarkers and diagnostic technologies to evaluate wound status and guide care.

Currently, wound closure is the only accepted objective endpoint to evaluate wound treatments, and there is a lack of quantitative surrogate biomarkers to predict healing at earlier stages. Multiphoton microscopy techniques, such as two-photon excited fluorescence (TPEF), have emerged as useful approaches to evaluate epithelial tissues and offer advantages in imaging depths and signal collection over confocal microscopy (Balu et al., 2013; Varone et al., 2014). Through TPEF imaging, the intrinsic fluorescence of nicotinamide and flavin adenine dinucleotides (NADH and FAD) can be measured without the application of exogenous stains (Georgakoudi and Quinn, 2012; Zipfel et al., 2003). An optical redox ratio of FAD/(NADH+FAD) autofluorescence has been computed from a variety of cells and tissues and is correlated with the intracellular concentration ratio of NAD^+/NADH (Quinn et al., 2013; Varone et al., 2014). This noninvasive measure of the relative rates of glucose catabolism to oxidative phosphorylation has been used to help diagnose disease and evaluate tissue development (Georgakoudi and Quinn, 2012).

Although metabolic stress at the systemic and tissue level often coincides with chronicity, the dynamics of metabolism in the wound at the cellular level are not well understood. Using label-free multiphoton microscopy, the objective of this study was to provide an initial quantitative assessment of metabolic biomarkers with sensitivity to differences between nondiabetic and diabetic wounds. To this end, multiphoton imaging was performed on unstained sections of full-thickness excisional wounds from the dorsum of a streptozotocin-induced diabetic mouse model. Animal studies were conducted in accordance with Beth Israel Deaconess Medical Center IACUC protocol #072-2012 and Tufts IACUC protocol #M2014-58. Additional methods'

Abbreviations: FAD, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; TPEF, two-photon excited fluorescence

Accepted manuscript published online 24 September 2015